THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 66:306–312, 1976

Vol. 66, No. 5
Printed in U.S.A.

IMPORTANCE OF PYRIMIDINE NUCLEOTIDE SALVAGE PATHWAYS FOR DNA SYNTHESIS IN SKIN

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Split-thickness rabbit skins were minced and incubated in vitro with radioactive precursors selected to measure de novo and salvage pathways for pyrimidine nucleotide synthesis. In this system, both the salvage precursors [^{3}H]thymidine and [^{14}C]cytidine were incorporated actively into skin DNA and only [^{14}C]cytidine into skin RNA. In contrast, the de novo precursor [^{14}C]orotic acid labeled skin RNA extensively but did not significantly label skin DNA. An unusually high ratio of specific activities of UMP:CMP in RNA was observed when [^{14}C]orotic acid was used as a nucleotide precursor.

Methotrexate effectively blocked thymidylate synthesis de novo but did not inhibit DNA synthesis as determined by [^{3}H]thymidine incorporation into DNA thymidylate residues or by [^{14}C]deoxycytidine incorporation into DNA deoxycytidylate residues. Azauridine inhibited labeling of RNA by [^{14}C]orotic acid to a greater extent than labeling of DNA by [^{3}H]thymidine.

These results suggest that in rabbit skin, cytidine nucleotides are utilized more effectively than uridine nucleotides for deoxypyrimidine biosynthesis, that DNA synthesis is primarily dependent upon salvage mechanisms to supply deoxypyrimidine nucleotides, and that inhibition of thymidylate synthesis by methotrexate does not inhibit DNA synthesis in this system.

The importance of salvage pathways for purine nucleotide biosynthesis in humans became strikingly apparent with the discovery of the Lesch–Nyhan syndrome in the mid 1960s [1]. This congenital neurologic disorder characterized by severe mental retardation and compulsive self-mutilation was shown to be associated with an almost complete lack of the purine salvage enzyme hypoxanthine/guanine phosphoribosyltransferase, along with a dramatic increase in purine nucleotide biosynthesis de novo in afflicted individuals [1–3]. Observations of the Lesch–Nyhan syndrome suggested that salvage pathways for nucleic acid biosynthesis as well as a regulatory balance between salvage and de novo routes are of crucial importance in mammalian metabolism.

Figure 1 outlines the de novo and salvage pathways for pyrimidine nucleotide synthesis in mammalian tissues. The first structure formed in the de novo pathway (*open arrows*) having the characteristic pyrimidine ring is orotic acid. It may be metabolized further, as shown, to uridine and cytidine nucleotide constituents of RNA as well as to thymidine and deoxycytidine nucleotide constituents of DNA. Pyrimidine nucleotide components of nucleic acids may also be acquired via salvage pathways (*solid arrows*) by incorporation of the nucelosides uridine and cytidine and the deoxynucleosides thymidine, deoxycytidine, and deoxyuridine.

Newborn mouse skin has been shown to carry out de novo pyrimidine nucleotide biosynthesis in vitro as well as to possess salvage mechanisms for incorporation of deoxypyrimidine nucleosides into DNA [4,5]. Although a knowledge of the relative contributions of salvage and de novo pathways to skin DNA synthesis could be of considerable value in developing new methods of chemotherapy for proliferative skin diseases, such information is not available for skin [6]; however, there are reports on

Manuscript received August 22, 1975; in revised form December 15, 1975; accepted for publication December 16, 1975.

This study was supported by Grant AM 10332 of the National Institutes of Health, United States Public Health Service.

Presented in part at the 32nd Annual Meeting of the American Federation for Clinical Research, Atlantic City, New Jersey, May 3, 1975.

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Abbreviations:

CDP: cytidine-5′-diphosphate
CMP: cytidine-5′-monophosphate
CTP: cytidine-5′-triphosphate
DHF: dihydrofolic acid
dCDP: deoxycytidine-5′-diphosphate
dCMP: deoxycytidine-5′-monophosphate
dCTP: deoxycytidine-5′-triphosphate
dTDP: deoxythymidine-5′-diphosphate
dTMP: deoxythymidine-5′-monophosphate
dTTP: deoxythymidine-5′-triphosphate
dUDP: deoxyuridine-5′-diphosphate
dUMP: deoxyuridine-5′-monophosphate
MTX: methotrexate
THF: tetrahydrofolic acid
UDP: uridine-5′-diphosphate
UMP: uridine-5′-monophosphate
UTP: uridine-5′-triphosphate

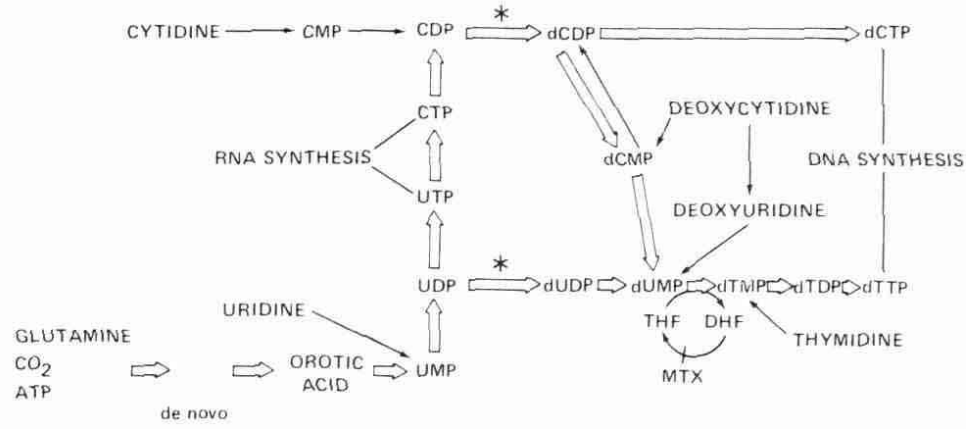

FIG. 1. Salvage and de novo pathways for pyrimidine ribonucleotide and deoxyribonucleotide biosynthesis. *Closed arrows* indicate salvage pathways; *open arrows* indicate de novo pathways. *Asterisks* indicate ribonucleotide reduction.

these pathways in other mammalian tissues [7–9]. Consequently, this investigation was undertaken to try to estimate the relative contributions of de novo and salvage pyrimidine nucleotide pathways to skin DNA synthesis by measuring the incorporation of radioactive pyrimidine nucleotide precursors into isolated ribonucleotides and deoxyribonucleotides of skin nucleic acids.

MATERIALS AND METHODS

Chemicals

[^{3}H]Thymidine (sp act 6 Ci/mmole), [^{14}C]orotic acid (sp act 54 mCi/mmole), [^{14}C]cytidine (sp act 28 mCi/mmole), [^{14}C]uridine (sp act 55 mCi/mmole), [^{14}C]deoxycytidine (sp act 54 mCi/mmole), L-aspartic acid, and L-glutamine were obtained from Schwarz/Mann. Pancreatic DNAase I (DN-CL), bacterial alkaline phosphatase, snake venom phosphodiesterase (Type VII), 6-azauridine, and hydroxyurea were purchased from Sigma. Methotrexate was purchased from Lederle Laboratories. Activated charcoal was obtained from Applied Science Laboratories. Bio-Gel P-2 (200–400 mesh) from Bio-Rad, and polyethyleneimine-impregnated cellulose thin-layer plates were purchased from Brinkmann Instruments. Double distilled water was used throughout.

Preparation of Tissue Minces

The skins of young adult rabbits were removed and the skins were shaved, rinsed in isotonic saline, cut to 0.2 mm thickness with a Castroviejo keratotome, rinsed in Hanks' balanced salt solution containing 100 units/ml of penicillin and 50 units/ml of streptomycin, blotted, and finely minced with scissors. Newborn mouse skins were removed, placed epidermis side down on a glass surface, and scraped free of fat and fascia with a scalpel. They were then rinsed and minced as for rabbit skin. Newborn mouse livers were removed, rinsed in Hanks' balanced salt solution as above, and were lightly blotted and minced with scissors.

Incubation of Skin Minces

Skin minces (400 mg/flask) were incubated in 2 ml (for newborn mouse skin) or 3 ml (for rabbit skin) of Hanks' balanced salt solution containing 50 units/ml of penicillin and 25 units/ml of streptomycin, 40 mM Tris buffer, pH 7.7, 1 mM D-ribose, 0.5 mM aspartic acid, 0.5 mM glutamine, and 0.5 mM sodium formate. Incubations were carried out for up to 4 hr under one atmosphere of O_2 at 37°C in a Dubnoff metabolic shaking incubator. When present, [^{3}H]thymidine was added at 1 μCi/ml of incubation medium with sufficient carrier thymidine to give a concentration of 9.8×10^{-6} M thymidine (sp act 102 mCi/mmole). [^{14}C]Deoxycytidine was present at 0.5 μCi/ml of incubation medium, [^{14}C]cytidine at 1 μCi/ml, [^{14}C]uridine at 2 μCi/ml, and [^{14}C]orotic acid was added at either 2 μCi/ml or 10 μCi/ml as indicated in the appropriate Table. All radioactive compounds and inhibitors were made up in incubation medium and were added to skin minces at the beginning of the incubation period. Relative specific activities of isolated nucleotides were determined by dividing the specific activity of the nucleotide (cpm/μmole) by the specific activity of its precursor (mCi/mmole).

Isolation and Hydrolysis of Skin Nucleic Acids

Following incubation, the incubation mixtures were made 0.5 N with respect to $HClO_4$, were homogenized with a Potter-Elvehjem glass homogenizer, chilled on ice for 20 to 30 min, and the precipitated macromolecules were collected by centrifugation at $1000 \times g$ in a refrigerated centrifuge. Acid precipitates were washed twice with cold 0.3 N $HClO_4$, once each with H_2O, 85%, 95%, and 100% ethanol and diethyl ether. Washed precipitates were allowed to dry at room temperature and were then hydrolyzed for 18 hr at 37°C in 0.5 N KOH to obtain ribonucleotides. DNA and protein were reprecipitated from alkaline hydrolysates with $HClO_4$ and were chilled on ice for 30 min prior to collection by centrifugation. The DNA plus protein precipitates were washed twice with cold 0.3 N $HClO_4$ and once with H_2O, and bulk DNA was obtained by extracting the precipitate twice with 0.5 N $HClO_4$ at 80°C for 20 min. Acid hydrolysates were neutralized and aliquots were removed for DNA determination by the method of Burton [10] and for liquid scintillation counting using Bray's cocktail [11]. Alkaline RNA hydrolysates were neutralized and aliquots were removed for scintillation counting and for RNA determination using the orcinol colorimetric method [12].

In order to hydrolyze DNA to its constituent deoxynucleotides, DNA plus protein precipitates were homogenized in 0.25 M $MgCl_2$ and the homogenates were adjusted to pH 7.6 with NaOH. DNAase I (0.5 mg) was added and samples were incubated for 2 hr at 37°C with periodic addition of NaOH to maintain the pH at 7.2 to 7.5. Following DNAase hydrolysis, the pH was adjusted to 8.2, 0.5 mg of phosphodiesterase was added, and hydrolysis was continued for an additional 2 hr while

maintaining the pH between 8.0 and 8.2. If deoxynucleosides were required, a further hydrolysis with 3 units of alkaline phosphatase for 2 hr at pH 8.2 was carried out. Following hydrolysis to either deoxynucleotides or deoxynucleosides, protein was precipitated by adjusting the pH to 2 with 2 N and 0.5 N $HClO_4$ and the precipitate was removed by centrifugation after chilling on ice for 30 min.

To convert ribonucleotides to the free bases, alkaline hydrolysates were neutralized with 4 N $HClO_4$, $KClO_4$ was removed by centrifugation after chilling on ice for 30 min, and the supernatant solutions were lyophilized to dryness. Residues were taken up in 1.2 ml of 70–72% $HClO_4$ and were boiled (100°C) for 1 hr. Hydrolysates were neutralized with KOH after dilution with 3 ml H_2O, $KClO_4$ was precipitated by chilling on ice for 30 min, and following centrifugation the supernatant solutions were lyophilized to dryness.

Chromatography of Ribonucleotides and Deoxyribonucleotides, Deoxyribonucleosides, and Free Bases

Charcoal columns 0.6 × 7.5 cm were washed with 0.1 N HCl, H_2O, characoal eluant (ethanol:H_2O:NH_3—125:122:3), H_2O, and 0.1 N HCl prior to use. Polyethyleneimine-impregnated cellulose thin-layer plates were washed and stored as described by Randerath and Randerath [13].

Alkaline hydrolysates containing ribonucleotides were adjusted to pH 2 and applied to prewashed charcoal columns which were then washed further with 200 ml H_2O followed by elution of ribonucleotides with 30 ml of charcoal eluant (see above). Eluted nucleotides were dried under vacuum and were dissolved in 0.3 ml H_2O. Aliquots (100 μl) were spotted on washed thin-layer plates and the plates were dipped in methanol for 15 min and dried prior to chromatography. Ribonucleotides were resolved by two-dimensional chromatography using the LiCl-HCOOH/NaCOOH procedure described by Colby and Edlin [14].

Purine and pyrimidine bases produced by strong acid hydrolysis of ribonucleotides were resolved on a 0.6 × 70 cm column of Bio-Gel P-2 [15].

Deoxynucleotides were applied to charcoal columns and eluted in the same way as described for ribonucleotides. Dried eluates were dissolved in 0.3 ml H_2O and 100 μl aliquots were applied to thin-layer plates. The plates were immersed in methanol for 5 min, dried, and chromatographed in 1 M LiCl saturated with boric acid (pH 4.5):ethanol—1:1 for resolution of the deoxynucleotides [16]. Deoxynucleosides were resolved into deoxypyrimidines and deoxypurines by column chromatography on Bio-Gel P-2 [15] without prior charcoal treatment.

Ribonucleotides and deoxyribonucleotides resolved by thin-layer chromatography were visualized under ultraviolet light. The nucleotides were marked, cut out of the plastic-backed plates, and were eluted from the cellulose with 0.1 N HCl at room temperature for 18 hr. Nucelotides were quantitated by ultraviolet absorption of the eluates at 260 and 280 nm. Compounds were identified by chromatographic position relative to known standards and by the ultraviolet absorption ratio of 280:260. After quantitation by ultraviolet absorption, eluates were dried under vacuum, dissolved in 0.5 ml H_2O, and were counted in Bray's solution in a Beckmann liquid scintillation spectrometer with a counting efficiency of 30% for ^3H and 60% for ^{14}C. Fractions collected from the Bio-Gel column (0.5 ml) were adjusted to 1.2 ml with H_2O, the ultraviolet absorption of each fraction at 260 nm was determined, and whole fractions were then counted in Bray's solution as above. Pyrimidine bases and deoxypyrimidine nucleosides were quantitated by the total absorbancy at 260 nm of Bio-Gel chromatographic peaks.

RESULTS

Time-Dependence of [^{3}H]Thymidine Incorporation into DNA

Figure 2 shows the time-dependent incorporation of [^{3}H]thymidine into rabbit skin DNA. Thymidine incorporation was linear for 3 hr and reached a plateau at 4 hr.

DNA and RNA Synthesis in Skin and Liver

Table I compares [^{3}H]thymidine and [^{14}C]orotic acid incorporation over a 3-hr interval into DNA and RNA of newborn mouse skin, newborn mouse liver, and adult rabbit skin. [^{3}H]Thymidine was actively incorporated into newborn mouse skin DNA and relatively little labeling of RNA preparations was observed. Although [^{14}C]orotic acid actively labeled newborn mouse skin RNA, very little labeling of skin DNA was obtained with this precursor. In contrast to skin under the same conditions in vitro, newborn mouse liver DNA and RNA were labeled when [^{14}C]orotic acid was used as a precursor. The difference between newborn mouse skin and liver with respect to [^{14}C]orotic acid labeling of DNA was more extensive than the difference in [^{3}H]thymidine incorporation between these two tissues. Like newborn mouse skin, rabbit skin incorporated [^{3}H]thymidine into DNA and [^{14}C]orotic acid into RNA, but did not exhibit substantial labeling of DNA by [^{14}C]orotate.

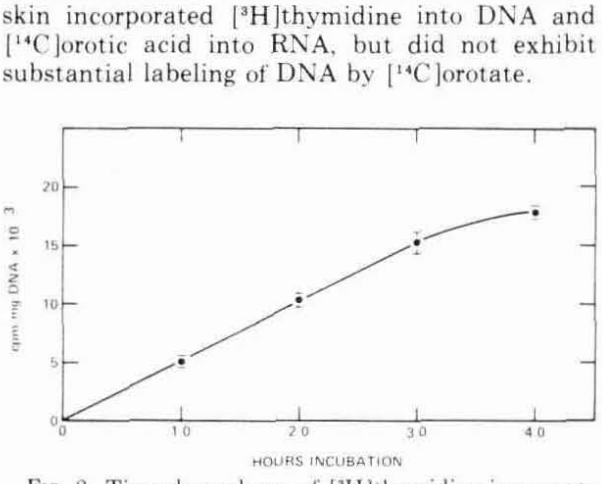

FIG. 2. Time dependence of [^{3}H]thymidine incorporation in vitro into rabbit skin DNA. Each point represents the mean value from two incubations.

TABLE I. *Incorporation of [^{3}H]thymidine and [^{14}C]orotic acid into skin and liver nucleic acids in vitro*

Tissue	Specific activity (cpm/μmole of DNA or RNA phosphate)			
	DNA		RNA	
	^{3}H	^{14}C	^{3}H	^{14}C
Newborn mouse liver	14,046	1,650	334	6,621
Newborn mouse skin	5,298	38	175	4,552
Adult rabbit skin	7,152	56	242	14,758

To determine whether the light labeling of rabbit skin DNA by [^{14}C]orotate represented actual incorporation into DNA at a low level or a small amount of RNA contamination of the DNA preparations, constituent deoxynucleosides were isolated by Bio-Gel P-2 column chromatography. Figure 3*a* shows the incorporation of [^{3}H]thymidine and [^{14}C]orotic acid into the deoxypyrimidine nucleosides (thymidine plus deoxycytidine) of rabbit skin DNA. A large peak of [^{3}H]thymidine incorporation (*open* triangles) was obtained along with a very small peak due to labeling of DNA by [^{14}C]orotate (*closed circles*). Figure 3*b* shows the extensive incorporation of [^{14}C]orotate into the deoxypyrimidine nucleosides of newborn mouse liver DNA (*closed circles*). No labeling of the deoxypurines was observed with either [^{3}H]thymidine or [^{14}C]orotic acid in liver or skin.

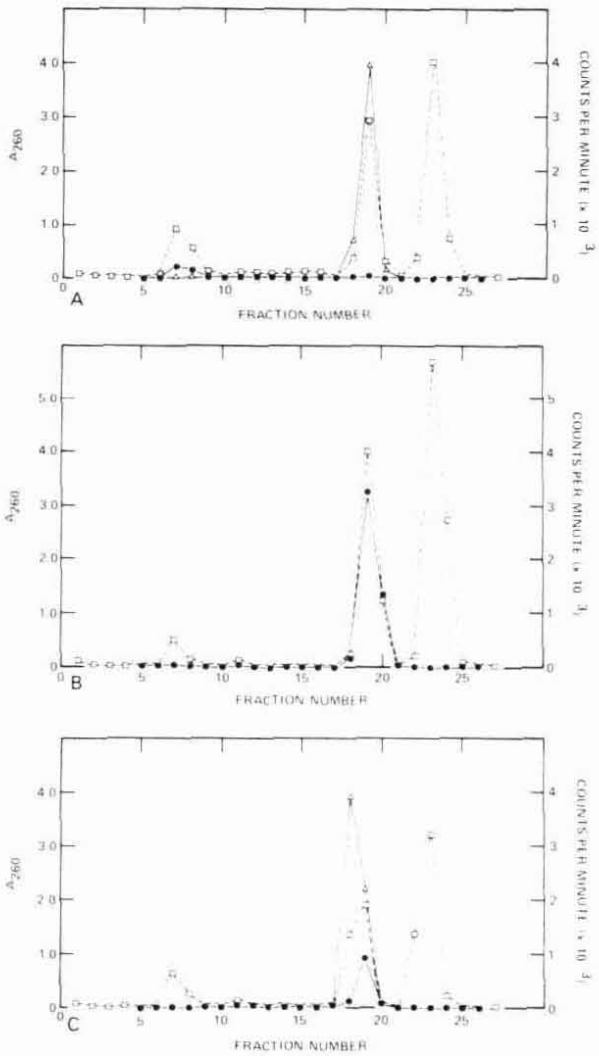

FIG. 3. Incorporation into DNA deoxypyrimidine nucleotides. *a*: [^{3}H]Thymidine and [^{14}C]orotic acid into rabbit skin. *b*: [^{14}C]Orotic acid into newborn mouse liver. *c*: [^{3}H]Thymidine and [^{14}C]cytidine into rabbit skin. Peak I (fraction numbers 6–9) represents a small amount of higher-molecular-weight nucleic acids and proteins; peak II (fraction numbers 17–20) represents isolated deoxypyrimidines (TdR plus CdR); peak III (fraction numbers 21–25) represents deoxypurines (AdR plus GdR). □——□ = Absorbancy at 260 nm; Δ——Δ = ^{3}H-cpm; ●——● = ^{14}C-cpm.

Incorporation of [^{14}C]Cytidine into Skin DNA and RNA

In contrast to the low level of labeling of DNA by orotic acid as shown in Figure 3*b*, the incorporation of cytidine into DNA was more extensive (Fig. 3*c*). Although the specific activity of [^{14}C]cytidine in these studies was lower than [^{14}C]orotic acid, the differences in uptake of isotopes was marked. Table II illustrates the magnitude of these differences. As shown in this Table, cytidine labels DNA 78-fold as extensively as orotic acid. Although [^{14}C]cytidine was incorporated into both UMP and CMP residues of RNA, orotic acid incorporation into RNA resulted in a relative specific activity of UMP 41 times that of CMP.

Incorporation of Nucleotide Precursors into Skin and Liver RNA and DNA

Table III compares the incorporation of labeled pyrimidine nucleotide precursors into rabbit skin and newborn mouse liver DNA and RNA. [^{3}H]Thymidine was omitted from these incuba-

TABLE II. *Incorporation of [^{3}H]thymidine and [^{14}C]orotic acid into rabbit skin pyrimidine nucleotides of RNA and deoxypyrimidine nucleosides of DNA*

Precursor	Relative specific activities			
	RNA ^{14}C		DNA Tdr + CdR	
	UMP	CMP	^{3}H	^{14}C
[^{14}C]Orotic acid + [^{3}H]thymidine	1,584	38	87	1
[^{14}C]Cytidine + [^{3}H]thymidine	4,226	15,982	122	78

TABLE III. *Incorporation of [^{14}C]labeled pyrimidine nucleotide precursors into rabbit skin and newborn mouse liver nucleic acids*

Tissue	Precursor	Relative specific activities		
		RNA		DNA
		UMP	CMP	CdR + TdR
Rabbit skin	[^{14}C]Orotate[a]	10,226	118	2
	[^{14}C]Uridine	28,167	298	9
	[^{14}C]Cytidine	8,190	30,323	90
Mouse liver	[^{14}C]Orotate	736	122	104
	[^{14}C]Cytidine	983	402	300

[a] [^{14}C]Orotic acid was present in this experiment at 10 μCi/ml resulting in a medium concentration 5-fold higher than that used for [^{14}C]cytidine or [^{14}C]uridine. In the mouse liver experiment, orotic acid concentration was the same as that for cytidine.

TABLE IV. *Effect of hydroxyurea and MTX on labeled deoxynucleoside incorporation into rabbit skin DNA*

Inhibitor	Precursor		Specific activity[a] (cpm/μmole deoxynucleotide) dTMP	dCMP
Hydroxyurea (100 μM)				
Ex. #1	[^{3}H]TdR	Control	27,460 ± 130	—
		Treated	15,306 ± 150	—
			(.005 < p < .01)[b]	
Ex. #2	[^{3}H]TdR	Control	13,619 ± 640	—
		Treated	7,531 ± 266	—
			(.001 < p < .005)	
Methotrexate (14 μM)				
Ex. #1	[^{3}H]TdR	Control	17,423 ± 964	—
		Treated	18,462 ± 2687	—
			(p > 0.5)	
Ex. #2	[^{14}C]CdR	Control	570 ± 88	3,187 ± 109
		Treated	73 ± 52	2,921 ± 253
			(.001 < p < .005)	(.05 < p < 0.1)
Ex. #3	[^{14}C]CdR	Control	1,918 ± 35	9,567 ± 923
		Treated	315 ± 68	9,707 ± 697
			(p < .001)	(p > 0.5)

[a] Specific activities are the mean values plus or minus standard deviation of 3 skin samples each for controls and inhibitor-treated preparations.

[b] p values calculated using Students *t*-test for paired data.

TABLE V. *Effect of azauridine on [^{14}C]orotic acid incorporation into rabbit skin RNA and upon [^{3}H]thymidine incorporation into skin DNA*

Azauridine concentration		Specific activity[a] RNA cpm/O.D. 260 Cytosine plus uracil	DNA cpm/μmole dTMP
Ex. 1. 5×10^{-5} M	Control	3,113 ± 604	18,541 ± 1,410
	Treated	1,760 ± 193	19,012 + 1,805
		(.05 < p < 0.1)[b]	(p > 0.5)
Ex. 2. 5×10^{-4} M	Control	3,195 ± 278	15,708 ± 450
	Treated	798 ± 92	12,525 ± 1,086
		(.005 < p < .01)	(.025 < p < .05)

[a] Specific activities are the mean plus or minus standard deviation of 3 skin samples each for control and inhibitor-treated preparations.

[b] p values calculated using Students *t*-test for paired data.

tions to rule out the possibility that thymidine might exert an inhibitory effect on DNA synthesis de novo as demonstrated by Cannon and Breitman [17]. In contrast to skin, newborn mouse liver exhibited a 3-fold difference in relative specific activities of deoxypyrimidines when cytidine and orotic acid were used as precursors. Incorporation of [^{14}C]orotic acid into newborn mouse liver and rabbit skin RNA was also different with respect to the relative labeling of uridylate and cytidylate residues. Labeling of newborn mouse liver RNA with [^{14}C]orotic acid resulted in a UMP:CMP ratio of relative specific activities of 6:1 compared to 86:1 for skin RNA. Similarly, [^{14}C]uridine incorporation into skin resulted in more extensive labeling of UMP than of CMP residues of RNA and labeled DNA to 1/10 the extent of [^{14}C]cytidine.

Effect of Hydroxyurea, Methotrexate, and Azauridine on Skin DNA and RNA Synthesis

Table IV shows the effects of hydroxyurea and MTX on incorporation of deoxynucleosides into rabbit skin DNA. Hydroxyurea, an inhibitor of skin ribonucleotide reductase (unpublished observations), caused a significant 44 to 45% inhibition of [^{3}H]thymidine incorporation into skin DNA.